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Award Number: DAMD17-01-1-0044

TITLE: A New Pathway of Chemotherapy Induced Apoptosis in a Prostate Cancer Cell Line

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REPORT DATE: June 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2003	Annual (1 Jun 02-31 May 03)

4. TITLE AND SUBTITLE
A New Pathway of Chemotherapy Induced Apoptosis in a Prostate Cancer Cell Line

5. FUNDING NUMBERS
DAMD17-01-1-0044

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

We have continued to systematically investigate several possibilities regarding the identity of caspase-P. Our systematic approach to chemotherapy-induced apoptosis helped us to advance the understanding of this process and to develop new tools to analyze mechanisms of cell death in tumor cells.

14. SUBJECT TERMS
Cancer, Apoptosis, Caspases

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The purpose of the study. Most cells, including cancer cells resistant to available drugs, retain the apoptotic machinery that can kill the cells within an hour. The cells are disassembled by caspases, a family of cysteine proteases. Caspases, together with their co-factors and inhibitors are organized in a protease cascade that can be initiated at several points by activating caspases known as initiators. Each initiator caspase is activated in response to a subset of cytotoxic signals. Therefore, the prerequisite to the understanding how a particular signal induces apoptosis is to identify the initiator caspase and to understand its regulation.

We found that apoptosis induced by chemotherapy in DU145, a prostate cancer cell line, required caspase activity, which was not surprising. However, we failed to assign this activity to a known caspase, and in particular to the caspases that have been thought to mediate chemotherapy-induced apoptosis. This suggested that apoptosis in this cell line is mediated by an unidentified caspase that we called caspase-P, or by a combination of known caspases that function simultaneously. Our preliminary results favored the first possibility. Hence, we hypothesized that chemotherapy can induce apoptosis through a yet unidentified pathway that is defined by caspase-P. Because dissecting other pathways of apoptosis gave insights into the mechanisms of chemotherapy, provided markers for drug-sensitivity, and suggested new ideas for drug design, we proposed to identify and characterize caspase-P and to begin learning its regulation.

The approaches. To identify caspase-P we proposed to exploit high affinity of caspases to p35, a viral caspase inhibitor. p35 inhibited chemotherapy-induced apoptosis in DU145 and was processed following drug treatment, which was consistent with the notion that p35 inhibits caspase-P. Because p35 forms highly stable complexes with inhibited caspases, we developed an approach to purify caspases by isolating the p35-caspase complexes. We also proposed alternative approaches, which included exploring putative caspases identified by the Human Genome Sequencing Project as caspase-P candidates.

BODY OF THE REPORT

What happened during the last year. As we described in the previous report, our strategy to identify caspase-P was modified by two developments. First, the completion of the human genome project provided a reasonable assurance that all caspases have been identified. Second, the development of the RNAi technology provided practical tools to silence gene expression in mammalian cells. Considering these developments, we decided to temporarily put aside our biochemical approach, and attempt to identify caspase-P by systematically silencing expression of all known caspases. The choice was made for two reasons. First, the new approach appeared to require much less effort and resources. In addition, we reasoned that exploiting new approaches may have several

"side" benefits, such as to verify our preliminary results obtained with the inhibitors of caspases and their dominant negative mutants, and to develop efficient and practical tools that, in principle, can be used as diagnostic tools to analyze apoptotic mechanisms in tumor cells. By the end of the first year, we did initial experiments with RNAi by optimizing transfection of siRNA into DU145 cells and using an siRNA to caspase-2 to test whether this caspase is involved in apoptosis. We found, however, that caspase-2 is not required for apoptosis in DU145 cells, even though these studies provided new insights into apoptosis in other cells.

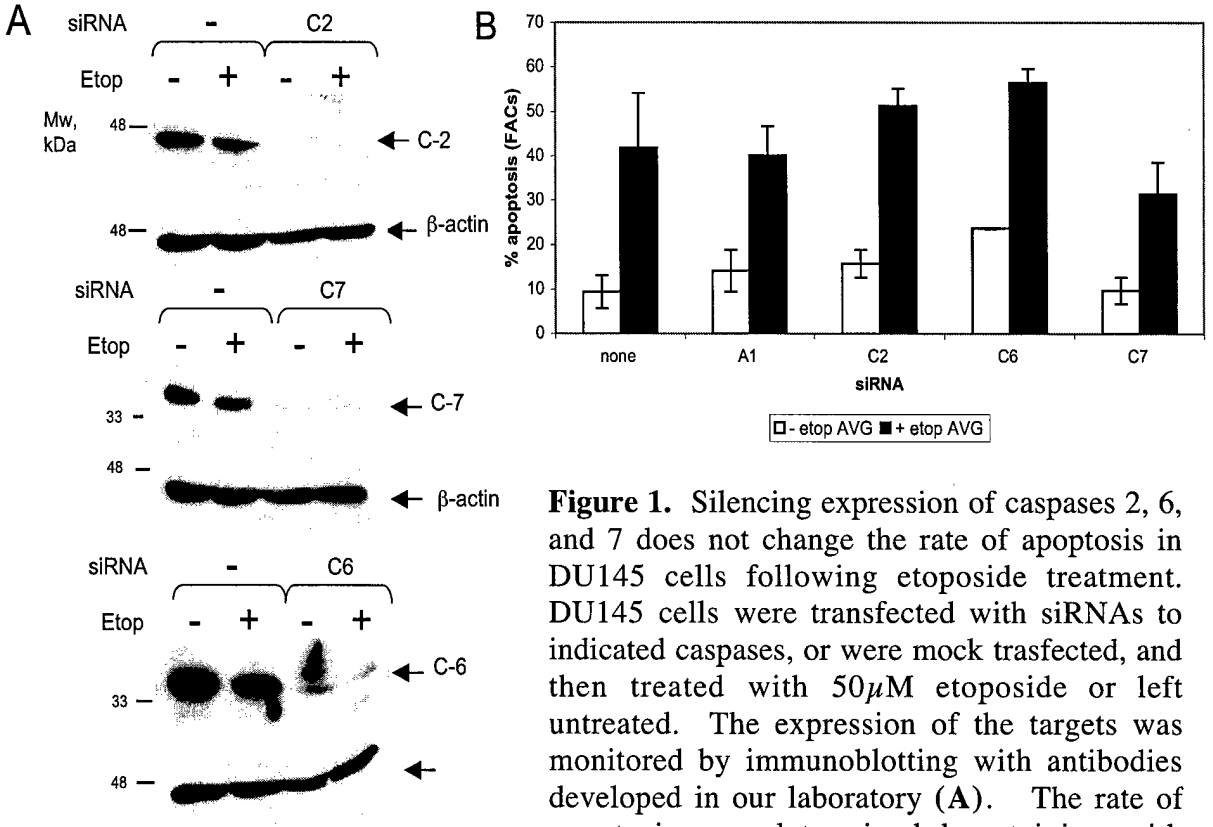

Figure 1. Silencing expression of caspases 2, 6, and 7 does not change the rate of apoptosis in DU145 cells following etoposide treatment. DU145 cells were transfected with siRNAs to indicated caspases, or were mock trasfected, and then treated with 50μM etoposide or left untreated. The expression of the targets was monitored by immunoblotting with antibodies developed in our laboratory (**A**). The rate of apoptosis was determined by staining with Annexin V-FITC with subsequent detection by flow cytometry **(B).**

During the last year we have designed siRNAs that efficiently silence Apaf-1, and caspases -4, -5, -6, and –7, and developed tools to monitor expression of caspases 4 and 5 (Figures 1 and 2). Because no specific inhibitors of either Apaf-1 or caspases 4, 5, 6, and 7 are available, RNAi provided a unique tool to directly test a requirement of these molecules for apoptosis. We found that none of these siRNAs prevented etoposide-induced apoptosis in DU145 cells, even though they efficiently silenced expression of their targets. These results confirmed our previous findings that the caspase-9 pathway is not required for drug-induced apoptosis in these cells. The finding that silencing caspases 4 and 5, which function is unknown, also had no effect (Figure 2), was consistent with our preliminary observation that crmA, a viral caspase inhibitor that was reported to inhibit these caspases, had no effect on etoposide induced apoptosis in

DU145. We are currently testing siRNAs to caspases 1, 8, 10, and 14, which would complete testing of all known caspases.

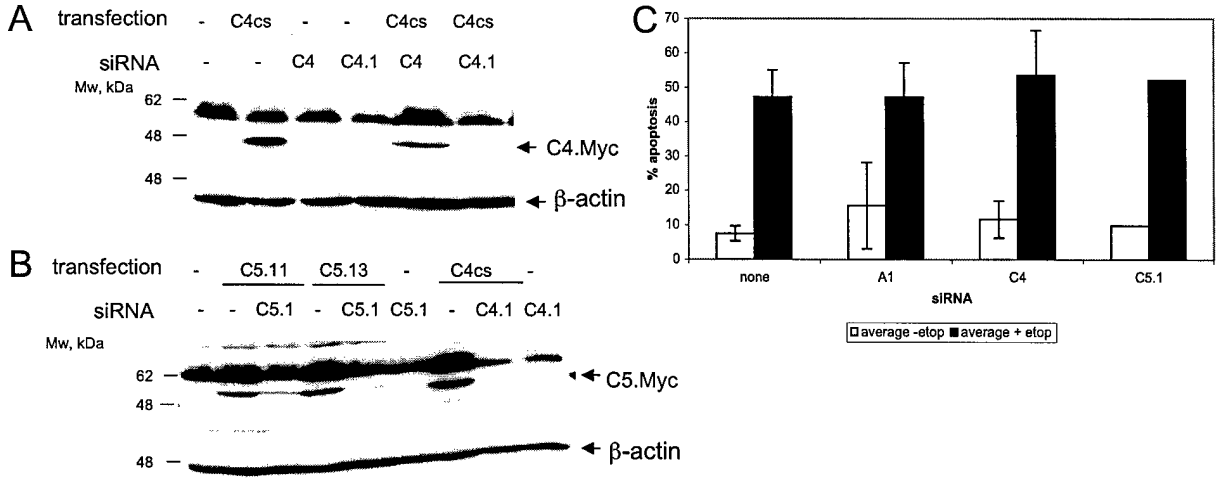

Figure 2. Silencing expression of caspases 4 and 5 has no effect of etoposide-induced apoptosis in DU145 cells. Because we found no reliable antibodies to caspases 4 and 5, we tested the efficiency of siRNA to these caspases by using ectopically expressed fusion of caspases 4 and 5 with a Myc epitope tag. We identified siRNAs to both caspases that efficiently inhibited expression of caspase-4-myc and caspase-5-myc (A, B). Our experience, and current knowledge about RNAi indicated that the endogenous target of an siRNA is silenced if the ectopically expressed protein is silenced. Therefore, we assumed that the siRNAs that we identified prevent expression of endogenous caspase-4 and caspase-5. We found no effect of this silencing on apoptosis, as detected by Annexin V staining with subsequent detection by flow cytometry (B). The experiments were done as described in Figure 2.

We are considering several explanations for our observations.

One is that caspase-P is among the caspases remained to be tested.

Another possibility is that several caspases are functioning simultaneously. For example, caspase-9 and caspase-2 have been shown to be compensatory in certain cases (Troy et al, 2001). Another possibility is that the caspase-9 and caspase-8 pathways function simultaneously, even though we previously found that expression of crmA, an inhibitor of caspase-8, and a dominant negative mutant of caspase-9 did not prevent apoptosis. We will address these possibilities by simultaneous silencing expression of two or more caspases. To make experiments more reproducible, we are currently adapting a technology that would allow us to make stable lines in which genes of interest are silenced by RNAi.

The third possibility is that caspase-P is not a caspase, despite the sensitivity of this activity to the caspase inhibitors p35 and ZVAD-FMK. Therefore, we are resuming experiments to purify proteins bound to p35. We will modify the approach by using cells in which expression of most abundant caspases, such as caspase-3 and 7, is silenced. We reason that this approach will facilitate purification of caspase-P by reducing the number of proteins that bind p35. As we outlined in the proposal, we are also setting up a cell free system that reproduces apoptosis in DU145 cells.

We are investigating these possibilities systematically as designed in our proposal, with the help of the new technologies and new information that became available after the study had began.

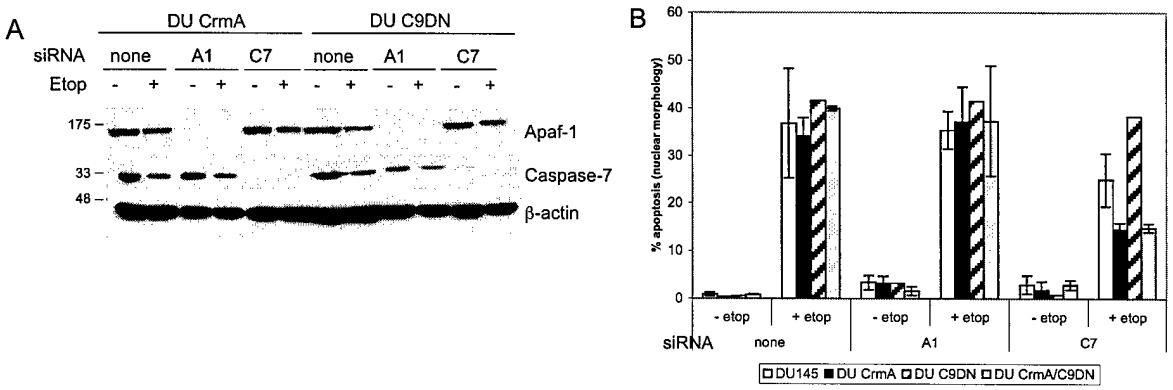

Figure 3. Etoposide-induced apoptosis in DU145 cells is inhibited by simultaneous expression of crmA and silencing of caspase-7 expression. DU145 (clear bars), DU145 expressing caspase-9 dominant negative (DU C9DN, striped bars), crmA (DU crmA, black bars), or both (DU crmA/C9DN, grey bars) were transfected with indicated siRNA or left untransfected, and then treated with etoposide for 16 hours or left untreated. Expression of the siRNA targets was monitored by immunoblotting (**A**, only DU crmA and DU C9DN are shown to avoid clutter), and apoptosis by counting cells with condensed chromatin (**B**). Note that, C9DN, crmA, or siRNA to caspase-7 have no effect on apoptosis. However, a combination of crmA and the siRNA inhibit apoptosis. This experiment was repeated five times with two independent lines of DU crmA, except that the treatment of DUC9DN was done only twice.

Since the original report was submitted, we have made some progress in testing the possibility that more than one caspase mediates apoptosis in DU145 cells. One of the approaches that we pursued was to silence expression of individual caspases in DU145 cells that express crmA (DU-crmA), an inhibitor of several caspases, or cells that express caspase-9 dominant negative, which inhibits activation of this caspase. We have found that silencing expression of caspase-7 prevented apoptosis in cells that express crmA (Figure 3). This funding is surprising, considering that caspase-7 is assumed to function as an executioner caspase like caspase-3. We repeated these experiments multiple times using two independent lines of DU-crmA. We are currently considering several explanations for our observation (Figure 4).

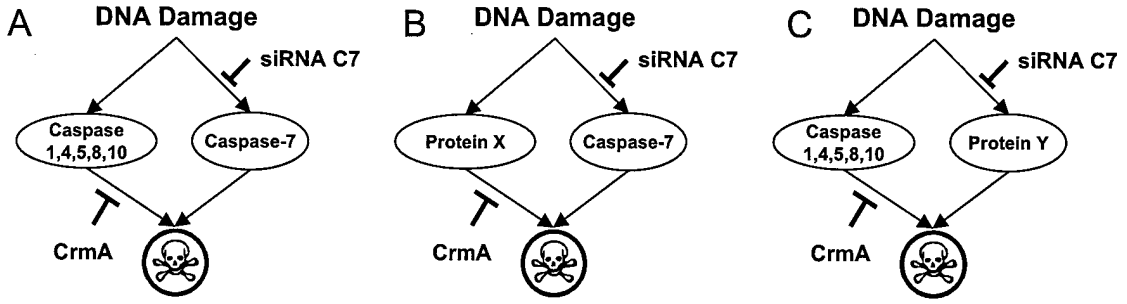

Figure 4. Models explaining the observation that crmA and the siRNA to caspase-7 synergize to prevent apoptosis in DU145 cells. All models assume that DNA damage induces more than one pathway. **A**. Both crmA and the siRNA eliminate the intended targets. This possibility implies that one of the caspases inhibitable by crmA is involved, which can be tested by silencing their expression one by one. **B**. CrmA inhibits a new target. This possibility will become plausible if silencing expression of known crmA targets will not mimic the effect of crmA. To find the protein inhibited by crmA, we will attempt to isolate molecules bound to this caspase inhibitor. **C**. siRNA to caspase-7 has an effect other than silencing of caspase-7 expression (an "off-target" effect). We are testing this possibility by investigating whether ectopic expression of caspase-7 can rescue the effect of the siRNA.

KEY RESEARCH ACCOMPLISHMENTS

We developed new tools to dissect pathways of apoptosis in tumor cells.

REPORTABLE OUTCOMES

The results of the study were reported at the following scientific meetings and invited seminars.

Meetings:

February 13-16, 2003. The 15th Lorne Cancer Conference, Lorne, Australia. Ttile "Oncogenic Transformation as an Activator of Apoptosis".

March 21-22, 2002. The National Institute of Child Health and Development workshop on apoptosis in the reproductive system, Bethesda, MD. The title "Mechanisms of apoptosis".

May 15-18, 2003. The Deutsche Forschungsgemeinschaft (DFG) annual meeting of the "Hinterzartener Kreis" for Cancer Research, Cadenabbia, Italy. Title "Oncogenic Transformation as an Activator of Apoptosis".

Seminars:

March 13, 2002. University of Vermont, Department of Pathology, Burlington, VT. The title "Oncogenes as a Trojan horse". Hosted by Dr. Ralph Budd.

October 2, 2002. University of Connecticut Center for Vascular Biology Seminar Series. Farmington, CT. Title "Oncogenic Transformation as a Cause of Cell Death". Hosted by Dr. Henry Furneaux.

October 22-23, 2002. M.D. Anderson Cancer Center, the Blaffer Series Seminars. Houston, TX. Title "Oncogenic Transformation as an Activator of Apoptosis" Hosted by Dr. Ryuji Kobayashi.

December 14, 2002. Karolinska Institutet, Stockholm, Sweden. Title "Biochemical mechanisms of apoptosis". Hosted by Dr. Sten Orrenius.

January 6, 2003. Tufts University, Department of Physiology, Boston, MA. Title "Apoptosis, cancer, and martial arts". Hosted by Dr. Irwin Arias.

March 14, 2003. National Cancer Institute, Genome Structure and Function Section in the Laboratory of Biosystems and Cancer. "Oncogenic transformation as a cause of apoptosis". Hosted by Dr. Vladimir Larionov.

March 17, 2003. Vanderbilt University, Department of Biochemistry, Nashville, TN. Ttile "Proteases in apoptosis". Hosted by Dr. Graham Carpenter.

April 15, 2003. James Graham Brown Cancer Center, University of Louisville, Louisville, Kentucky. Title "Cell death, martial arts, and a radio". Hosted by Dr. Jason Chesney.

May 22, 2003. University of Medicine and Dentistry of New Jersey, Department of Biochemistry. Newark, NJ. Ttile "Oncogenic transformation as a cause of apoptosis". Hosted by Dr. Raymond Birge.

CONCLUSIONS

Our systematic approach to chemotherapy-induced apoptosis helped us to advance the understanding of this process and to develop new tools to analyze mechanisms of cell death in tumor cells.